

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> C12N 15/11, 15/62, 5/16, 15/54	<b>A1</b>	<b>(11) International Publication Number:</b> WO 98/05768 <b>(43) International Publication Date:</b> 12 February 1998 (12.02.98)
<b>(21) International Application Number:</b> PCT/AU97/00492 <b>(22) International Filing Date:</b> 1 August 1997 (01.08.97)  <b>(30) Priority Data:</b> PO 1402 2 August 1996 (02.08.96) AU 60/024,279 21 August 1996 (21.08.96) US  <b>(71) Applicant (for all designated States except US):</b> THE AUSTIN RESEARCH INSTITUTE [AU/AU]; Kronheimer Building, The Austin Hospital, Studley Road, Heidelberg, VIC 3084 (AU).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> McKENZIE, Ian, Farquhar, Campbell [AU/AU]; 359 Brunswick Road, Brunswick, VIC 3056 (AU). SANDRIN, Mauro, Sergio [AU/AU]; 211 Barkley Street, Brunswick, VIC 3056 (AU).  <b>(74) Agent:</b> SANTER, Vivien; Griffith Hack, 509 St. Kilda Road, Melbourne, VIC 3004 (AU).		<b>(81) Designated States:</b> AU, CA, JP, SG, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IMPROVED NUCLEIC ACIDS ENCODING A CHIMERIC GLYCOSYLTRANSFERASE  <b>(57) Abstract</b>  The invention relates to nucleic acids which encode glycosyltransferase and are useful in producing cells and organs from one species which may be used for transplantation into a recipient of another species. It also relates to the production of nucleic acids which, when present in cells of a transplanted organ, result in reduced levels of antibody recognition of the transplanted organ.		

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>C12N 15/11, 15/62, 5/16, 15/54</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 98/05768</b> <b>(43) International Publication Date:</b> 12 February 1998 (12.02.98)
<b>(21) International Application Number:</b> PCT/AU97/00492 <b>(22) International Filing Date:</b> 1 August 1997 (01.08.97)  <b>(30) Priority Data:</b> PO 1402 ✓ 2 August 1996 (02.08.96) AU 60/024,279 ✓ 21 August 1996 (21.08.96) US  <b>(71) Applicant (for all designated States except US):</b> THE AUSTIN RESEARCH INSTITUTE [AU/AU]; Kronheimer Building, The Austin Hospital, Studley Road, Heidelberg, VIC 3084 (AU).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> McKENZIE, Ian, Farquhar, Campbell [AU/AU]; 359 Brunswick Road, Brunswick, VIC 3056 (AU). SANDRIN, Mauro, Sergio [AU/AU]; 211 Barkley Street, Brunswick, VIC 3056 (AU).  <b>(74) Agent:</b> SANTER, Vivien; Griffith Hack, 509 St. Kilda Road, Melbourne, VIC 3004 (AU).		<b>(81) Designated States:</b> AU, CA, JP, SG, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IMPROVED NUCLEIC ACIDS ENCODING A CHIMERIC GLYCOSYLTRANSFERASE  <b>(57) Abstract</b>  The invention relates to nucleic acids which encode glycosyltransferase and are useful in producing cells and organs from one species which may be used for transplantation into a recipient of another species. It also relates to the production of nucleic acids which, when present in cells of a transplanted organ, result in reduced levels of antibody recognition of the transplanted organ.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

IMPROVED NUCLEIC ACIDS ENCODING A CHIMERIC  
GLYCOSYLTRANSFERASE

Field of the Invention

5           The present invention relates to nucleic acids  
which encode glycosyltransferase and are useful in  
producing cells and organs from one species which may be  
used for transplantation into a recipient of another  
species. Specifically the invention concerns production of  
10   nucleic acids which, when present in cells of a  
transplanted organ, result in reduced levels of antibody  
recognition of the transplanted organ.

Background of the Invention

15           The transplantation of organs is now practicable,  
due to major advances in surgical and other techniques.  
However, availability of suitable human organs for  
transplantation is a significant problem. Demand outstrips  
supply. This has caused researchers to investigate the  
20   possibility of using non-human organs for transplantation.

Xenotransplantation is the transplantation of  
organs from one species to a recipient of a different  
species. Rejection of the transplant in such cases is a  
particular problem, especially where the donor species is  
25   more distantly related, such as donor organs from pigs and  
sheep to human recipients. Vascular organs present a  
special difficulty because of hyperacute rejection (HAR).

30           HAR occurs when the complement cascade in the  
recipient is initiated by binding of antibodies to donor  
endothelial cells.

35           Previous attempts to prevent HAR have focused on  
two strategies : modifying the immune system of the host by  
inhibition of systemic complement formation (1,2), and  
antibody depletion (3,4). Both strategies have been shown  
to prolong xenograft survival temporarily. However, these  
methodologies are therapeutically unattractive in that they  
are clinically impractical, and would require chronic

- 2 -

immunosuppressive treatments. Therefore, recent efforts to inhibit HAR have focused on genetically modifying the donor xenograft. One such strategy has been to achieve high-level expression of species-restricted human complement inhibitory proteins in vascularized pig organs via transgenic engineering (5-7). This strategy has proven to be useful in that it has resulted in the prolonged survival of porcine tissues following antibody and serum challenge (5,6). Although increased survival of the transgenic tissues was observed, long-term graft survival was not achieved (6). As observed in these experiments and also with systemic complement depletion, organ failure appears to be related to an acute antibody-dependent vasculitis (1,5).

In addition to strategies aimed at blocking complement activation on the vascular endothelial cell surface of the xenograft, recent attention has focused on identification of the predominant xenogeneic epitope recognised by high-titre human natural antibodies. It is now accepted that the terminal galactosyl residue, Gal- $\alpha$ (1,3)-Gal, is the dominant xenogeneic epitope (8-15). This epitope is absent in Old World primates and humans because the  $\alpha$ (1,3)-galactosyltransferase (gal-transferase or GT) is non-functional in these species. DNA sequence comparison of the human gene to  $\alpha$ (1,3)-galactosyltransferase genes from the mouse (16,17), ox (18), and pig (12) revealed that the human gene contained two frameshift mutations, resulting in a non-functional pseudogene (20,21). Consequently, humans and Old World primates have pre-existing high-titre antibodies directed at this Gal- $\alpha$ (1,3)-Gal moiety as the dominant xenogeneic epitope.

One strategy developed was effective to stably reduce the expression of the predominant Gal- $\alpha$ (1,3)-Gal epitope. This strategy took advantage of an intracellular competition between the gal-transferase and  $\alpha$ (1,2)-fucosyltransferase (H-transferase) for a common acceptor substrate. The gal-transferase catalyses the transfer of a

- 3 -

terminal galactose moiety to an N-acetyl lactosamine acceptor substrate, resulting in the formation of the terminal Gal- $\alpha$ (1,3)-Gal epitope. Conversely, H-transferase catalyses the transfer of a fucosyl residue to the N-acetyl lactosamine acceptor substrate, and generates a fucosylated N-acetyl lactosamine (H-antigen, i.e., the O blood group antigen), a glycosidic structure that is universally tolerated. Although it was reported that expression of human H-transferase transfected cells resulted in high level expression of the non-antigenic H-epitope and significantly reduced the expression of the Gal- $\alpha$ (1,3)-Gal xenoepitope, there are still significant levels of Gal- $\alpha$ (1,3)-Gal epitope present on such cells.

#### Summary of the Invention

In view of the foregoing, it is an object of the present invention to further reduce levels of undesirable epitopes in cells, tissues and organs which may be used in transplantation.

In work leading up to the invention the inventors surprisingly discovered that the activity of H transferase may be further increased by making a nucleic acid which encodes a H transferase catalytic domain but is anchored in the cell at a location where it is better able to compete for substrate with gal transferase. Although work by the inventors focused on a chimeric H transferase, other glycosyltransferase enzymes may also be produced in accordance with the invention.

Accordingly, in a first aspect the invention provides a nucleic acid encoding a chimeric enzyme, wherein said chimeric enzyme comprises a catalytic domain of a first glycosyltransferase and a localisation signal of a second glycosyltransferase, whereby when said nucleic acid is expressed in a cell said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with a second glycosyltransferase, resulting in reduced levels of a product from said second

glycosyltransferase.

Preferably the nucleic acid is in an isolated form; that is the nucleic acid is at least partly purified from other nucleic acids or proteins.

5            Preferably the nucleic acid comprises the correct sequences for expression, more preferably for expression in a eukaryotic cell. The nucleic acid may be present on any suitable eukaryotic expression vector such as pcDNA (Invitrogen). The nucleic acid may also be present on other  
10 vehicles whether suitable for eukaryotes or not, such as plasmids, phages and the like.

            Preferably the catalytic domain of the first glycosyltransferase is derived from H transferase, secretor sialyltransferase, a galactosyl sulphating enzyme or a  
15 phosphorylating enzyme.

            The nucleic acid sequence encoding the catalytic domain may be derived from, or similar to a glycosyltransferase from any species. Preferably said species is a mammalian species such as human or other  
20 primate species, including Old World monkeys, or other mammals such as ungulates (for example pigs, sheep, goats, cows, horses, deer, camels) or dogs, mice, rats and rabbits. The term "similar to" means that the nucleic acid is at least partly homologous to the glycosyltransferase  
25 genes described above. The term also extends to fragments of and mutants, variants and derivatives of the catalytic domain whether naturally occurring or man made.

            Preferably the localisation signal is derived from a glycosyltransferase which produces glycosylation  
30 patterns which are recognised as foreign by a transplant recipient. More preferably the localisation signal is derived from  $\alpha(1,3)$  galactosyltransferase. The effect of this is to downregulate the level of Gal- $\alpha(1,3)$ -Gal produced in a cell when the nucleic acid is expressed by  
35 the cell.

            The nucleic acid sequence encoding the localisation signal may be derived from any species such as

- 5 -

those described above. Preferably it is derived from the same species as the cell which the nucleic acid is intended to transform i.e., if pig cells are to be transformed, preferably the localization signal is derived from pig.

5 More preferably the nucleic acid comprises a nucleic acid sequence encoding the catalytic domain of H transferase and a nucleic acid sequence encoding a localisation signal from Gal transferase. Still more preferably both nucleic acid sequences are derived from  
10 pigs. Even more preferably the nucleic acid encodes gtHT described herein.

The term "nucleic acid" refers to any nucleic acid comprising natural or synthetic purines and pyrimidines. The nucleic acid may be DNA or RNA, single or  
15 double stranded or covalently closed circular.

The term "catalytic domain" of the chimeric enzyme refers to the amino acid sequences necessary for the enzyme to function catalytically. This comprises one or more contiguous or non-contiguous amino acid sequences.  
20 Other non-catalytically active portions also may be included in the chimeric enzyme.

The term "glycosyltransferase" refers to a polypeptide with an ability to move carbohydrates from one molecule to another.

25 The term "derived from" means that the catalytic domain is based on, or is similar, to that of a native enzyme. The nucleic acid sequence encoding the catalytic domain is not necessarily directly derived from the native gene. The nucleic acid sequence may be made by polymerase  
30 chain reaction (PCR), constructed *de novo* or cloned.

The term "localisation signal" refers to the amino acid sequence of a glycosyltransferase which is responsible for anchoring it in location within the cell. Generally localisation signals comprise amino terminal  
35 "tails" of the enzyme. The localisation signals are derived from a second glycosyltransferase, the activity of which it is desired to minimise. The localisation of a



- 6 -

catalytic domain of a first enzyme in the same area as the second glycosyltransferase means that the substrate reaching that area is likely to be acted on by the catalytic domain of the first enzyme, enabling the amount of substrate catalysed by the second enzyme to be reduced.

The term "area of the cell" refers to a region, compartment or organelle of the cell. Preferably the area of the cell is a secretory organelle such as the Golgi apparatus.

In another aspect the invention provides an isolated nucleic acid molecule encoding a localisation signal of a glycosyltransferase. Preferably the signal encoded comprises an amino terminus of said molecule; more preferably it is the amino terminus of gal transferase. The gal transferase may be derived from or based on a gal transferase from any mammalian species, such as those described above. Particularly preferred sequences are those derived from pig, mouse or cattle.

In another aspect the invention relates to a method of producing a nucleic acid encoding a chimeric enzyme, said enzyme comprising a catalytic domain of a first glycosyltransferase and a localisation signal of a second glycosyltransferase whereby when said nucleic acid is expressed in a cell said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with a second glycosyltransferase said method comprising operably linking a nucleic acid sequence encoding a catalytic domain from a first glycosyltransferase to a nucleic acid sequence encoding a localisation signal of a second glycosyltransferase.

The term "operably linking" means that the nucleic acid sequences are ligated such that a functional protein is able to be transcribed and translated.

Those skilled in the art will be aware of various techniques for producing the nucleic acid. Standard techniques such as those described in Sambrook et al may be employed.

- 7 -

Preferably the nucleic acid sequences are the preferred sequences described above.

In another aspect the invention provides a method of reducing the level of a carbohydrate exhibited on the surface of a cell, said method comprising causing a nucleic acid to be expressed in said cell wherein said nucleic acid encodes a chimeric enzyme which comprises a catalytic domain of a first glycosyltransferase and a localisation signal of a second glycosyltransferase, whereby said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with said second glycosyltransferase, and wherein said second glycosyltransferase is capable of producing said carbohydrate.

The term "reducing the level of a carbohydrate" refers to lowering, minimising, or in some cases, ablating the amount of carbohydrate displayed on the surface of the cell. Preferably said carbohydrate is capable of stimulating recognition of the cell as "non-self" by the immune system of an animal. The reduction of such a carbohydrate therefore renders the cell, or an organ composed of said cells, more acceptable to the immune system of a recipient animal in a transplant situation or gene therapy situation.

The term "causing a nucleic acid to be expressed" means that the nucleic acid is introduced into the cell (i.e. by transformation/transfection or other suitable means) and contains appropriate signals to allow expression in the cells.

The cell may be any suitable cell, preferably mammalian, such as that of a New World monkey, ungulate (pig, sheep, goat, cow, horse, deer, camel, etc.) or other species such as dogs.

In another aspect the invention provides a method of producing a cell from one species (the donor) which is immunologically acceptable to another species (the recipient) by reducing levels of carbohydrate on said cell

- 8 -

which cause it to be recognised as non-self by the other species, said method comprising causing a nucleic acid to be expressed in said cell wherein said nucleic acid encodes a chimeric enzyme which comprises a catalytic domain of a first glycosyltransferase and a localisation signal of a second glycosyltransferase, whereby said chimeric enzyme is located in an area of the cell where it is able to compete for substrate with said second glycosyltransferase, and wherein said second glycosyltransferase is capable of producing said carbohydrate.

The term "immunologically acceptable" refers to producing a cell, or an organ made up of numbers of the cell, which does not cause the same degree of immunological reaction in the recipient species as a native cell from the donor species. Thus the cell may cause a lessened immunological reaction, only requiring low levels of immunosuppressive therapy to maintain such a transplanted organ or no immunosuppression therapy.

The cell may be from any of the species mentioned above. Preferably the cell is from a New World primate or a pig. More preferably the cell is from a pig.

The invention extends to cells produced by the above method and also to organs comprising the cells.

The invention further extends to non-human transgenic animals harbouring the nucleic acid of the invention. Preferably the species is a human, ape or Old World monkey.

The invention also extends to the proteins produced by the nucleic acid. Preferably the proteins are in an isolated form.

In another aspect the invention provides an expression unit which expresses the nucleic acid of the invention, resulting in a cell which is immunologically acceptable to an animal having reduced levels of a carbohydrate on its surface, which carbohydrate is recognised as non-self by said species. In a preferred embodiment, the expression unit is a retroviral packaging

- 9 -

cell, cassette, a retroviral construct or retroviral producer cell.

Preferably the species is a human, ape or Old World monkey.

5           The retroviral packaging cells or retroviral producer cells may be cells of any animal origin where it is desired to reduce the level of carbohydrates on its surface to make it more immunologically acceptable to a host. Such cells may be derived from mammals such as  
10 canine, rodent or ruminant species and the like.

The retroviral packaging and/or producer cells may be used in applications such as gene therapy. General methods involving use of such cells are described in PCT/US95/07554 and the references discussed therein.

15           The invention also extends to a method of producing a retroviral packaging cell or a retroviral producer cell having reduced levels of a carbohydrate on its surface wherein the carbohydrate is recognised as non-self by a species, comprising transforming/transfecting a  
20 retroviral packaging cell or a retroviral producer cell with the nucleic acid of the invention under conditions such that the chimeric enzyme is produced.

#### Brief Description of the Drawings

25           Figure 1 Schematic diagram of normal and chimeric glycosyltransferases

The diagram shows normal glycosyltransferases porcine  $\alpha(1,3)$ galactosyltransferase (GT) and human  
30  $\alpha(1,2)$ fucosyltransferase (HT), and chimeric transferases ht-GT in which the cytoplasmic domain of GT has been completely replaced by the cytoplasmic domain of HT, and gt-HT in which the cytoplasmic domain of HT has been entirely replaced by the cytoplasmic domain of GT. The  
35 protein domains depicted are cytoplasmic domain CYTO, transmembrane domain TM, stem region STEM, catalytic domain CATALYTIC. The numbers refer to the amino acid sequence of

- 10 -

the corresponding normal transferase.

Figure 2 Cell surface staining of COS cells transfected with normal and chimeric transferases

5 Cells were transfected with normal GT or HT or with chimeric transferases gt-HT or ht-GT and 48h later were stained with FITC-labelled lectin IB4 or UEAI. Positive-staining cells were visualised and counted by fluorescence microscopy. Results are from at least three  
10 replicates and values are +/- SEM.

Figure 3. RNA analysis of transfected COS cells

Northern blots were performed on total RNA prepared from COS cells transfected: Mock, mock-  
15 transfected; GT, transfected with wild-type GT; GT1-6/HT, transfected with chimeric transferase gt-HT; GT1-6/HT + HT1-8/GT, co-transfected with both chimeric transferases gt-HT and ht-GT; HT1-8/GT, transfected with chimeric transferase ht-GT; HT, transfected with normal HT; GT + HT,  
20 co-transfected with both normal transferases GT and HT. Blots were probed with a cDNA encoding GT (Top panel), HT (Middle panel) or g-actin (Bottom panel).

Figure 4. Enzyme kinetics of normal and chimeric glycosyltransferases

25 Lineweaver-Burk plots for  $\alpha(1,3)$  galactosyltransferase ( $\square$ ) and  $\alpha(1,2)$  fucosyltransferase ( $\blacksquare$ ) to determine the apparent  $K_m$  values for N-acetyl lactosamine. Experiments were performed in triplicate,  
30 plots shown are of mean values of enzyme activity of wild-type transferases, GT and HT, and chimeric proteins ht-GT and gt-HT in transfected COS cell extracts using phenyl-B-D Gal and N-acetyl lactosamine as acceptor substrates.

Figure 5. Staining of cells co-transfected with chimeric transferases

35 Cells were co-transfected with cDNAs encoding

- 11 -

normal transferases GT + HT (panels A, B), with chimeric transferases gt-HT + ht-GT (panels C, D), with HT + ht-GT (panels E, F) or with GT + gt-HT (panels G, H) and 48h later were stained with FITC-labelled lectin IB4 (panels A, C, E, G) or UEA1 (panels B, D, F, H).

Figure 6 is a representation of the nucleic acid sequence and corresponding amino acid sequence of pig secretor.

Figure 7 is a representation of the nucleic acid sequence and corresponding amino acid sequence of pig H.

Figure 8 Cell surface staining of pig endothelial cell line (PIEC) transfected with chimeric  $\alpha(1,2)$ -fucosyltransferase. Cells were transfected and clones exhibiting stable integration were stained with UEA1 lectin and visualised by fluorescence microscopy.

Figure 9 Screening of chimeric  $\alpha(1,2)$ -fucosyltransferase transferase in mice. Mice were injected with chimeric  $\alpha(1,2)$ -fucosyltransferase and the presence of the transferase was analysed by dot blots.

#### Description of the Preferred Embodiment

The nucleic acid sequences encoding the catalytic domain of a glycosyltransferase may be any nucleic acid sequence such as those described in PCT/US95/07554, which is herein incorporated by reference, provided that it encodes a functional catalytic domain with the desired glycosyltransferase activity.

Preferred catalytic domains from glycosyltransferase include H transferase and secretor. Preferably these are based on human or porcine sequences.

The nucleic acid sequences encoding the localisation signal of a second transglycosylase may be any nucleic acid sequence encoding a signal sequence such as signal sequences disclosed in P A Gleeson, R D Teasdale &

- 12 -

J Bourke, Targeting of proteins to the Golgi apparatus. Glycoconjugate J. (1994) 11: 381-394. Preferably the localisation signal is specific for the Golgi apparatus, more preferably for that of the trans Golgi. Still more preferably the localisation signal is based on that of Gal transferase. Even more preferably the localisation signal is based on porcine, murine or bovine sequences. Even more preferably the nucleic acid encodes a signal sequence with following amino acid sequence (in single letter code):

10 MNVKGR (porcine), MNVKGK (mouse) or MVVKGK (bovine).

Vectors for expression of the chimeric enzyme may be any suitable vector, including those disclosed in PCT/US95/07554.

15 The nucleic acid of the invention can be used to produce cells and organs with the desired glycosylation pattern by standard techniques, such as those disclosed in PCT/US95/07554. For example, embryos may be transfected by standard techniques such as microinjection of the nucleic acid in a linear form into the embryo (22). The embryos are  
20 then used to produce live animals, the organs of which may be subsequently used as donor organs for implantation.

Cells, tissues and organs suitable for use in the invention will generally be mammalian cells. Examples of suitable cells and tissues such as endothelial cells,  
25 hepatic cells, pancreatic cells and the like are provided in PCT/US95/07554.

The invention will now be described with reference to the following non-limiting Examples.

- 13 -

## ABBREVIATIONS

The abbreviations used are bp, base pair(s); FITC, fluorescein isothiocyanate; GT, galactosyltransferase; H substance,  $\alpha(1,2)$ fucosyl lactosamine; HT,  $\alpha(1,2)$ fucosyltransferase; PCR, polymerase chain reaction; Example 1 Cytoplasmic domains of glycosyltransferases play a central role in the temporal action of enzymes

## EXPERIMENTAL PROCEDURES

Plasmids - The plasmids used were prepared using standard techniques (7); pGT encodes the cDNA for the porcine  $\alpha(1,3)$ galactosyltransferase (23), pHT encodes the cDNA for the  $\alpha(1,2)$ fucosyltransferase (human) (25). Chimeric glycosyltransferase cDNAs were generated by polymerase chain reaction as follows: an 1105 bp product ht-GT was generated using primers corresponding to the 5' end of ht-GT (5'-GCGGATCCATGTGGCTCCGGAGCC ATCGTCAGGTGGTTCTGTCAATGC TGCTTG-3') coding for nucleotides 1-24 of HT (25) followed immediately by nucleotides 68-89 of GT (8) and containing a BamH1 site (underlined) and a primer corresponding to the 3' end of ht-GT (5'-GCTCTAGAGCGTCAGATGTTATT TCTAACCAAATTATAC-3') containing complementarity to nucleotides 1102-1127 of GT with an Xba1 site downstream of the translational stop site (underlined); an 1110 bp product gt-HT was generated using primers corresponding to the 5' end of gt-HT (5'-GCGGATCCATGAATGTCAAAGGAAGACTCTGCCTGGCCT TCCTGC-3') coding for nucleotides 49-67 of GT followed immediately by nucleotides 25-43 of HT and containing a BamH1 site (underlined) and a primer corresponding to the 3' end of gt-HT (5'-GCTCTAGAGCCTCAAGGCTTAG CCAATGTCCAGAG-3') containing complementarity to nucleotides 1075-1099 of HT with a Xba1 site downstream of the translational stop site (underlined). PCR products were restricted BamH1/Xba1, gel-purified and ligated into a BamH1/Xba1 digested pcDNA1



- 14 -

expression vector (Invitrogen) and resulted in two plasmids pht-GT (encoding the chimeric glycosyltransferase ht-GT) and pgt-HT (encoding the chimeric glycosyltransferase gt-HT) which were characterised by restriction mapping,

5 Southern blotting and DNA sequencing .

Transfection and Serology - COS cells were maintained in Dulbecco's modified Eagles Medium (DMEM) (Trace Biosciences Pty. Ltd. , Castle Hill, NSW, Australia) and were transfected (1-10 µg DNA/5 x 10<sup>5</sup> cells) using DEAE-Dextran (26); 48h later cells were examined for cell surface expression of H substance or Gal-α(1,3)-Gal using FITC-conjugated lectins: IB4 lectin isolated from Griffonia simplicifolia (Sigma, St. Louis, MO) detects Gal-α(1,3)-Gal (27); UEA1 lectin isolated from Ulex europaeus (Sigma, St. Louis, MO) detects H substance (28). H substance was also detected by indirect immunofluorescence using a monoclonal antibody (mAb) specific for the H substance (ASH-1952) developed at the Austin Research Institute, using FITC-conjugated goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA) to detect mAb binding. Fluorescence was detected by microscopy.

RNA Analyses - Cytoplasmic RNA was prepared from transfected COS cells using RNazol (Biotechx Laboratories, Houston, TX), and total RNA was electrophoresed in a 1% agarose gel containing formaldehyde, the gel blotted onto a nylon membrane and probed with random primed GT or HT cDNA.

Glycosyltransferase assays - Forty-eight hours after transfection, cells were washed twice with phosphate buffered saline and lysed in 1% Triton X-100/ 100 mM cacodylate pH 6. 5/ 25 mM MnCl<sub>2</sub>, at 4°C for 30 min; lysates were centrifuged and the supernatant collected and stored at -70°C. Protein concentration was determined by the Bradford method using bovine serum albumin as standard (29). Assays for HT activity (30) were performed in 25 µl containing 3mM [GDP-<sup>14</sup>C]fucose (specific activity 287 mCi/mmol, Amersham International), 5mM ATP, 50mM MOPS pH 6. 5, 20 mM MnCl<sub>2</sub>, using 2-10 µl of cell extract

- 15 -

(approximately 15-20 $\mu$ g of protein) and a range of concentrations (7.5 -75 mM) of the acceptor phenyl-B-D-galactoside (Sigma). Samples were incubated for 2h at 37°C and reactions terminated by the addition of ethanol and water. The amount of  $^{14}$ C-fucose incorporated was counted after separation from unincorporated label using Sep-Pak C18 cartridges (Waters-Millipore, Millford, MA). GT assays (31) were performed in a volume of 25  $\mu$ l using 3mM UDP[ $^3$ H]-Gal (specific activity 189mCi/mmol, Amersham International), 5mM ATP, 100mM cacodylate pH 6.5, 20mM MnCl<sub>2</sub> and various concentrations (1 -10 mM) of the acceptor N-acetyl lactosamine (Sigma). Samples were incubated for 2h at 37°C and the reactions terminated by the addition of ethanol and water.  $^3$ H-Gal incorporation was counted after separation from non-incorporated UDP[ $^3$ H]-Gal using Dowex I anion exchange columns (BDH Ltd. , Poole, UK) or Sep-Pak Accell plus QMA anion exchange cartridges (Waters-Millipore, Millford, MA). All assays were performed in duplicate and additional reactions were performed in the absence of added acceptor molecules, to allow for the calculation of specific incorporation of radioactivity.

## RESULTS

### Expression of chimeric $\alpha(1,3)$ galactosyltransferase and $\alpha(1,2)$ fucosyltransferase cDNAs

We had previously shown that when cDNAs encoding  $\alpha(1,3)$ galactosyltransferase (GT) and  $\alpha(1,2)$ fucosyltransferase (HT) were transfected separately they could both function efficiently leading to expression of the appropriate carbohydrates: Gal- $\alpha(1,3)$ -Gal for GT and H substance for HT (32). However when the cDNAs for GT and HT were transfected together, the HT appeared to "dominate" over the GT in that H substance expression was normal, but Gal- $\alpha(1,3)$ -Gal was reduced. We excluded trivial reasons for this effect and considered that the localisation of the enzymes may be the reason. Thus, if the HT localisation signal placed the enzyme in an earlier temporal compartment

- 16 -

than GT, it would have "first use" of the N-acetyl  
lactosamine substrate. However, such a "first use" if it  
occurred, was not sufficient to adequately reduce GT. Two  
chimeric glycosyltransferases were constructed using PCR  
5 wherein the cytoplasmic tails of GT and HT were switched.  
The two chimeras constructed are shown in Fig.1: ht-GT  
which consisted of the NH<sub>2</sub> terminal cytoplasmic tail of HT  
attached to the transmembrane, stem and catalytic domains  
of GT; and gt-HT which consisted of the NH<sub>2</sub> terminal  
10 cytoplasmic tail of GT attached to the transmembrane, stem  
and catalytic domains of HT. The chimeric cDNAs were  
subcloned into the eukaryotic expression vector pcDNA1 and  
used in transfection experiments.

The chimeric cDNAs encoding ht-GT and gt-HT were  
15 initially evaluated for their ability to induce  
glycosyltransferase expression in COS cells, as measured by  
the surface expression of the appropriate sugar using  
lectins. Forty-eight hours after transfection COS cells  
were tested by immunofluorescence for their expression of  
20 Gal- $\alpha$ (1,3)-Gal or H substance (Table 1 & Fig. 2). The  
staining with IB4 (lectin specific for Gal- $\alpha$ (1,3)-Gal) in  
cells expressing the chimera ht-GT (30% of cells stained  
positive) was indistinguishable from that of the normal GT  
staining (30%) (Table 1 & Fig. 2). Similarly the intense  
25 cell surface fluorescence seen with UEA1 staining (the  
lectin specific for H substance) in cells expressing gt-HT  
(50%) was similar to that seen in cells expressing wild-  
type pHT (50%) (Table 1 & Fig. 2). Furthermore, similar  
levels of mRNA expression of the glycosyltransferases GT  
30 and HT and chimeric glycosyltransferases ht-GT and gt-HT  
were seen in Northern blots of total RNA isolated from  
transfected cells (Fig. 3). Thus both chimeric  
glycosyltransferases are efficiently expressed in COS cells  
and are functional indeed there was no detectable  
35 difference between the chimeric and normal  
glycosyltransferases.

- 17 -

Glycosyltransferase activity in cells transfected with chimeric cDNAs encoding ht-GT and gt-HT

To determine whether switching the cytoplasmic tails of GT and HT altered the kinetics of enzyme function, we compared the enzymatic activity of the chimeric glycosyltransferases with those of the normal enzymes in COS cells after transfection of the relevant cDNAs. By making extracts from transfected COS cells and performing GT or HT enzyme assays we found that N-acetyl lactosamine was galactosylated by both GT and the chimeric enzyme ht-GT (Fig 4. panel A) over a the 1-5mM range of substrate concentrations. Lineweaver-Burk plots showed that both GT and ht-GT have a similar apparent Michealis-Menten constant of  $K_m$  2. 6mM for N-acetyl lactosamine (Fig. 4. panel B). Further HT, and the chimeric enzyme gt-HT were both able to fucosylate phenyl-B-D-galactoside over a range of concentrations (7. 5 - 25 mM) (Fig. 4 panel C) with a similar  $K_m$  of 2. 3mM (Fig. 4 panel D), in agreement with the reported  $K_m$  of 2. 4mM for HT (25). Therefore the chimeric glycosyltransferases ht-GT and gt-HT are able to utilise N-acetyl lactosamine (ht-GT) and phenyl-B-D-galactoside (gt-HT) in the same way as the normal glycosyltransferases, thus switching the cytoplasmic domains of GT and HT does not alter the function of these glycosyltransferases and if indeed the cytoplasmic tail is the localisation signal then both enzymes function as well with the GT signal as with the HT signal.

Switching cytoplasmic domains of GT and HT results in a reversal of the "dominance" of the glycosyltransferases

The cDNAs encoding the chimeric transferases or normal transferases were simultaneously co-transfected into COS cells and after 48h the cells were stained with either IB4 or UEA1 lectin to detect Gal- $\alpha$ (1,3)-Gal and H substance respectively on the cell surface (Table 1 & Fig. 5). COS cells co-transfected with cDNAs for ht-GT + gt-HT (Fig 5 panel C) showed 30 % cells staining positive with IB4

- 18 -

(Table 1) but no staining on cells co-transfected with cDNAs for GT + HT (3%) (Fig. 5 panel A). Furthermore staining for H substance on the surface of ht-GT + gt-HT co-transfectants gave very few cells staining positive (5%) (Fig 5 panel D) compared to the staining seen in cells co-transfected with cDNAs for the normal transferases GT + HT (50%) (Fig. 5 panel B), ie. the expression of Gal- $\alpha$ (1,3)-Gal now dominates over that of H. Clearly, switching the cytoplasmic tails of GT and HT led to a complete reversal in the glycosylation pattern seen with the normal transferases i.e. the cytoplasmic tail sequences dictate the pattern of carbohydrate expression observed.

That exchanging the cytoplasmic tails of GT and HT reverses the dominance of the carbohydrate epitopes points to the glycosyltransferases being relocalized within the Golgi. To address this question, experiments were performed with cDNAs encoding glycosyltransferases with the same cytoplasmic tail: COS cells transfected with cDNAs encoding HT + ht-GT stained strongly with both UEA1 (50%) and IB4 (30%) (Table 1 & Fig. 5 panels E, F), the difference in staining reflecting differences in transfection efficiency of the cDNAs. Similarly cells transfected with cDNAs encoding GT + gt-HT also stained positive with UEA1 (50%) and IB4 (30%) (Table 1 & Fig. 5 panel G, H). Thus, glycosyltransferases with the same cytoplasmic tail leads to equal cell surface expression of the carbohydrate epitopes, with no "dominance" of one glycosyltransferase over the other observed, and presumably the glycosyltransferases localised at the same site appear to compete equally for the substrate.

In COS cells the levels of transcription of the cDNAs of chimeric and normal glycosyltransferases were essentially the same (Fig.3) and the immunofluorescence pattern of COS cells expressing the chimeric glycosyltransferases ht-GT and gt-HT showed the typical staining pattern of the cell surface Gal- $\alpha$ (1,3)-Gal and H substance respectively (Table 1 & Fig. 2), the pattern

b ing indistinguishable from that of COS cells expressing normal GT and HT. Our studies showed that the  $K_m$  of ht-GT for N-acetyl lactosamine was identical to the  $K_m$  of GT for this substrate, similarly the  $K_m$  of gt-HT for  
5 phenylBDgalactoside was approximately the same as the  $K_m$  of HT for phenylbDgalactoside (Fig. 3). These findings indicate that the chimeric enzymes are functioning in a cytoplasmic tail-independent manner, such that the catalytic domains are entirely functional, and are in  
10 agreement with those of Henion et al (23), who showed that an  $NH_2$  terminal truncated marmoset GT (including truncation of the cytoplasmic and transmembrane domains) maintained catalytic activity and confirmed that GT activity is indeed independent of the cytoplasmic domain sequence.

15 If the Golgi localisation signal for GT and HT is contained entirely within the cytoplasmic domains of the enzymes, then switching the cytoplasmic tails between the two transferases should allow a reversal of the order of glycosylation. Co-transfection of COS cells with cDNA  
20 encoding the chimeric glycosyltransferases ht-GT and gt-HT caused a reversal of staining observed with the wild type glycosyltransferases (Fig. 5), demonstrating that the order of glycosylation has been altered by exchanging the cytoplasmic tails. Furthermore, co-transfection with cDNA  
25 encoding glycosyltransferases with the same cytoplasmic tails (i. e. HT + ht-GT and GT + gt-HT) gave rise to equal expression of both Gal- $\alpha$ (1,3)-Gal and H substance (Fig.5). The results imply that the cytoplasmic tails of GT and HT are sufficient for the localisation and retention of these  
30 two enzymes within the Golgi.

To date only twenty or so of at least one hundred predicted glycosyltransferases have been cloned and few of these have been studied with respect to their Golgi  
localisation and retention signals (34). Studies using the  
35 elongation transferase N-acetylglucosaminyltransferase I (33-37), the terminal transferases  $\alpha$ (2,6)sialyltransferase (24-26) and  $\beta$ (1,4)galactosyltransferase (38-40) point to

- 20 -

residues contained within the cytoplasmic tail, transmembrane and flanking stem regions as being critical for Golgi localisation and retention. There are several examples of localisation signals existing within cytoplasmic tail domains of proteins including the KDEL and KKXX motifs in proteins resident within the endoplasmic reticulum (41,42) the latter motif also having been identified in the cis Golgi resident protein ERGIC-53 (43) and a di-leucine containing peptide motif in the mannose-6-phosphate receptor which directs the receptor from the trans-Golgi network to endosomes (44). These motifs are not present within the cytoplasmic tail sequences of HT or GT or in any other reported glycosyltransferase. To date a localisation signal in Golgi resident glycosyltransferases has not been identified and while there is consensus that transmembrane domains are important in Golgi localisation, it is apparent that this domain is not essential for the localisation of all glycosyltransferases, as shown by the study of Munro (45) where replacement of the transmembrane domain of  $\alpha(2,6)$ sialyltransferase in a hybrid protein with a poly-leucine tract resulted in normal Golgi retention. Dahdal and Colley (46) also showed that sequences in the transmembrane domain were not essential to Golgi retention. This study is the first to identify sequence requirements for the localisation of  $\alpha(1,2)$ fucosyltransferase and  $\alpha(1,3)$ galactosyltransferase within the Golgi. It is anticipated that other glycosyltransferases will have similar localisation mechanisms.

Example 2                      Use of secretor in construction of a  
chimeric enzyme

A construct is made using PCR and subcloning as described in Example 1, such that amino acids #1 to #6 of the pig  $\alpha(1,3)$ -galactosyltransferase (MNVKGR) replace amino acids #1 to 5 of the pig secretor (Fig 6). Constructs are tested as described in Example 1.

Example 3            Use of pig H transferase in construction of a chimeric enzyme

5        A construct is made using PCR and subcloning as described in Example 1, such that amino acids #1 to #6 of the pig  $\alpha(1,3)$ -galactosyltransferase (MNVKGR) replace amino acids #1 to 8 of the pig H transferase (Fig 7). Constructs are tested as described in Example 1.

10      Example 4.            Generation of pig endothelial cells expressing chimeric  $\alpha(1,2)$ fucosyltransferase

         The pig endothelial cell line PIEC expressing the chimeric  $\alpha(1,2)$ fucosyltransferase was produced by lipofectamine transfection of pgtHT plasmid DNA (20  $\mu$ g) and  
15      pSV2NEO (2  $\mu$ g) and selecting for stable integration by growing the transfected PIEC in media containing G418 (500  $\mu$ g/ml; Gibco-BRL, Gaithersburg, MD). Fourteen independent clones were examined for cell surface expression of H substance by staining with UEA-1 lectin. >95% of cells of  
20      each of these clones were found to be positive. Fig. 8 shows a typical FACS profile obtained for these clones.

Example 5            Production of transgenic mice expressing chimeric  $\alpha(1,2)$ fucosyltransferase

25        A NruI/NotI DNA fragment, encoding the full length chimeric  $\alpha(1,2)$ fucosyltransferase, was generated utilising the Polymerase Chain Reaction and the pHHT plasmid using the primers:  
5' primer homologous to the 5'UTR:  
30      5'-TTCGCGAATGAATGTCAAAGGAAGACTCTG, in which the underlined sequence contains a unique NruI site;  
3' primer homologous to the 3'UTR:  
5'-GGCGGCCGCTCAGATGTTATTTCTAACCAAAT  
the underlined sequence contains a NotI site

35        The DNA was purified on gels, electroeluted and subcloned into a NruI/NotI cut genomic H-2Kb containing vector resulting in the plasmid clone (pH-2Kb-gtHT)



- 22 -

encoding the chimeric  $\alpha(1,2)$ -fucosyltransferase gene directionally cloned into exon 1 of the murine H-2Kb gene, resulting in a transcript that commences at the H-2Kb transcriptional start site, continuing through the gtHT cDNA insert. The construct was engineered such that translation would begin at the initiation codon (ATG) of the hHT cDNA and terminate at the in-phase stop codon (TGA).

DNA was prepared for microinjection by digesting pH-2Kb-hHT with XhoI and purification of the H-2Kb-hHT DNA from vector by electrophoretic separation in agarose gels, followed by extraction with chloroform, and precipitation in ethanol to decontaminate the DNA. Injections were performed into the pronuclear membrane of (C57BL/6xSJL)F1 zygotes at concentrations between 2-5ng/ml, and the zygotes transferred to pseudopregnant (C57BL/6xSJL)F1 females.

The presence of the transgene in the live offspring was detected by dot blotting. 5mg of genomic DNA was transferred to nylon filters and hybridized with the insert from gtHT, using a final wash at 68°C in 0.1xSSC/1% SDS. Fig. 9 shows the results of testing 12 live offspring, with two mice having the transgenic construct integrated into the genome. Expression of transgenic protein is examined by estimating the amount of UEAI lectin (specific for H substance) or anti-H mAb required to haemagglutinate red blood cells from transgenic mice. Hemagglutination in this assay demonstrates transgene expression.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

References cited herein are listed on the following pages, and are incorporated herein by this

reference.

- 24 -

TABLE 1

EXPRESSION OF GAL- $\alpha$ (1,3)GAL AND H SUBSTANCE BY COS CELLS  
 TRANSFECTED WITH cDNAs ENCODING NORMAL AND CHIMERIC  
 5 GLYCOSYLTRANSFERASES

COS cells transfected with cDNA encoding:	%IB4 positive cells	%UEAI positive cells
GT	30	0
HT	0	50
ht-GT	30	0
gt-HT	3	50
GT+HT	3	50
ht-GT+gt-HT	33	5
GT+gt-HT	30	30
GT+ht-GT	30	0
HT+ht-GT	30	30
HT+gt-HT	0	50
Mock	0	0

Transfected COS cells were stained with FITC-labelled IB4  
 (lectin specific for Gal- $\alpha$ (1,3)Gal or UEAI (lectin specific  
 10 for H substance) and positive staining cells were  
 visualized and counted by fluorescence microscopy. Results  
 are from at least three replicates.

## REFERENCES

1. Leventhal, J R et al. Complement depletion  
prolongs discordant cardiac xenograft survival in rodents  
5 nad non-human primates. Transplantn Prod. 25, 398-399  
(1993).
2. Pruitt, S et al. The effect of soluble complement  
receptor type 1 on hyperacute rejection of porcine  
xenografts. Transplantation 57, 363-370 (1994).
- 10 3. Leventhal, J R et al. Removal of baboon and human  
antiporcine IgG and IgM natural antibodies by  
immunoabsorption. Transplantation 59, 294-300 (1995).
4. Brewer, R J et al. Depletion of preformed natural  
antibody in primates for discordant xenotransplantation by  
15 continuous donor organ plasma perfusion. Transplantation  
Proac. 25, 385-386 (1993).
5. McCurry, K R et al. Human complement regulatory  
proteins protect swine-to-primate cardiac xenografts from  
humoral injury. Nature Med. 1, 423-427 (1995).
- 20 6. Fodor, W L et al. Expression of a functional  
human complement inhibitor in a transgenic pig as a model  
for the prevention of xenogeneic hyperacute organ  
rejection. Proc. Natn. Acad. Sci USA 91, 11153-11157  
(1994).
- 25 7. Rosengard, A M et al. Tissue expression of the  
human complement inhibitor decay accelerating factor in  
transgenic pigs. Transplantation 59, 1325-1333 (1995).
8. Sandrin, M S, Vaughan, H A, Dabkowski, P L &  
McKenzie, I F C. Anti-pig IgM antibodies in human serum  
30 reacts predominantly with Gal(a1,3)Gal epitopes. Prod.  
Natn. Acad. Sci USA 90, 11391-11395 (1993).
9. Sandrin, M S, Vaughan, H A & McKenzie, I F C.  
Identification of Gal(a1,3)Gal as the major epitope of pig-  
to-human vascularised xenografts. Transplantation Rev. 8,  
35 134-149 (1994).
10. Sandrin, M S & McKenzie, I F C. Gal(a1,3)Gal, the  
major xenoantigen(s) recognised in pigs by human natural

antibodies. Immunol. Rev. 141. 169-190 (1994).

11. Cooper, D K C et al. Identification of a-galactosyl and other carbohydrate epitopes that are bound by human anti-pig antibodies. Relevance to discordant xenografting in man. Transplantation Immun. 1. 198-205 (1993).

12. Cooper, D K C, Koren, E & Oriol, R. Oligosaccharides and discordant xenotransplantation. Immunol. Rev. 141. 31-58 (1994).

13. Good, A H et al. Identification of carbohydrate structures that bind antiporcine antibodies: Implications for discordant xenografting in humans. Transplantation Proc. 24. 559-562 (1992).

14. Galili, U., Clark, M R., Shohet, S B., Buehler, J & Macher, B A. Evolutionary relationship between the natural anti-Gal antibody and the Gal $\alpha$ 1-3Gal epitope in primates. Proc. Natn. Acad. Sci USA 84. 1369-1373 (1987).

15. Galili, U., Shohet, S B., Korbin, E., Stults, C L M & Macher, B A. Man, apes and Old world monkeys differ from other mammals in the expression of the a-galactosyl epitopes on nucleated cells. J. biol. Chem. 263. 17755-17762 (1988).

16. Larsen, R D et al. Isolation of a cDNA encoding a murine UDPgalactose:b-D-galactosyl-1, 4-N-acetylglucosaminide-1,3-galactosyltransferase: Expression cloning by gene transfer. Proc. natn. Acad. Sci. USA 86. 8227-8231d (1989).

17. Joziassse, D H., Shaper, J H., Kim D., Van den Eijnden, D H & Shaper, J H. Murine  $\alpha$ 1,3 galactosyltransferase a single gene locus specifies four isoforms of the enzyme by alternative splicing. J. biol. Chem. 267, 5534-5541 (1992).

18. Joziassse, D H, Shaper, J H, Van den Eijnden, D H, Van Tunen, A J & Shaper, N L. bovine  $\alpha$ 1,3 galactosyltransferase: Isolation and characterization of a cDNA clone. Identification of homologous sequences in human genomic DNA. J. biol. Chem. 264. 14290-14297 (1989).

19. Sandrin, M S, Dabkowski, P I, Henning, M M,  
Mouhtouris, E & McKenzie,  
I F C. Characterization of cDNA clones for  
porcine  $\alpha 1,3$  galactosyltransferase. The enzyme generating  
the Gal( $\alpha 1,3$ )Gal epitope. Xenotransplantation 1, 81-88  
5 (1994).
20. Joziassse, D H, Shaper, J H, Jabs, F W & Shaper, N  
L. Characterization of an  $\alpha 1,3$ -galactosyltransferase  
homologue on human chromosome 12 that is organized as a  
processed pseudogene. J. biol. Chem. 266. 6991-6998 (1991).  
10
21. Larsen, R D, Riverra-Marrero, C A, Ernst, L K,  
Cummings, R D & Lowe, J B. Frameshift and non sense  
mutations in a human genomic sequence homologous to a murine  
UDP-Gal:b-D-Gal 1,4-D- GlcNAc  $\alpha 1,3$ -galactosyl-transferase  
15 cDNA. J. biol. Chem. 265. 7055-7061 (1990).
22. Kiote, C et al. Introduction of  $\alpha(1,2)$ -  
fucosyltransferase and its effect on  $\alpha$ -Gal epitopes in  
transgenic pig. Xenotransplantation 3:81-86.
23. Sandrin, M. S., Dabkowski, P. L., Henning, M. M.,  
20 Mouhtouris, E., and McKenzie, I. F. C. (1994)  
Xenotransplantation 1, 81-88
24. Cohney, S., Mouhtouris, E., McKenzie, I. F. C.,  
and Sandrin, M. S. (1996) Immunogenetics 44(1), 76-79
25. Larsen, R. D., Ernst, L. K., Nair, R. P., and  
25 Lowe, J. B. (1990) Proc. Natl. Acad. Sci. USA 87, 6674-6678
26. Sandrin, M. S., Vaughan, H. A., Dabkowski, P. L.,  
and McKenzie, I. F. C. (1993) Proc. Natl. Acad. Sci. USA  
90, 11391-11395
27. Hayes, C. E., and Goldstein, I. J. (1974) J.  
30 Biol. Chem. 6, 1904-1914
28. Matsumoto, I., and Osowa, T. (1969) Biochim.  
Biophys. Acta 194, 180-189
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
30. Rajan, V. R., Larsen, R. D., Ajmera, S., Ernst,  
35 L. K., and Lowe, J. B. (1989) J. Biol. Chem 264, 11158-  
11167
31. Van der Eijnden, D. H., Blanken, W. M.,

Winterwerp, H., and Schiphorst, W. E. C. M. (1983) Eur. J. Biochem. 134, 523-530

32. Sandrin, M. S., Fodor, W. F., Mouhtouris, E., Osman, N., Cohney, S. C., Rollins, S. A., Guilmette, E. R.,  
5 Setter, E., Squinto, S. P., and McKenzie, I. F. C. (1995) Nature Med. 1, 1261-1267

33. Henion, T. R., Macher, B. A., Anaraki, F., and Galili, U. (1994) Glycobiology 4, 193-201

34. Schachter, H. (1994) in Molecular Glycobiology  
10 (Fukuda, M., and Hindsgaul, O., eds), pp. 88-162, Oxford University Press, Oxford

35. Burke, J., Pettitt, J. M., Schachter, H., Sarkar, M., and Gleeson, P. A. (1992) J. Biol. Chem. 267, -24433-24440

15 36. Tang, B. L., Wong, S. H., Low, S. H., and Hong, W. (1992) J. Biol. Chem. 267, 10122

37. Nilsson, T., Pyper, M., Hoe, M. H., Slusarewicz, P., Berger, E., and Warren, G. (1993) J. Cell Biol. 120, 5-

20 38. Nilsson, T., Lucocq, J. M., Mackay, D., and Warren, G. (1991) EMBO J. 10, 3567-3575

39. Aoki, D., Lee, N., Yamaguchi, N., Dubois, C., and Fukuda, M. N. (1992) Proc. natl. Acad. Sci. USA 89, 4319-4323

25 40. Teasdale, R. D., D'Agostaro, G. D., and Gleeson, P. A. (1992) J. Biol. Chem. 267, 4084-4096

41. Pelham, H. R. (1990) Trends Biochem. Sci. 15, 483-486

42. Jackson, M. R., Nilsson, T., and Peterson, P. A.  
30 (1990) EMBO J. 9, 3153-3162

43. Kappeler, F., Itin, C., Schindler, R., and Hauri, H.-P. (1994) J. Biol. Chem. 269, 6279-6281

44. Johnson, K. F., and Kornfeld, S. (1992) J. Biol. Chem. 267, 17110-17115

35 45. Munro, S. (1991) EMBO J. 10, 3577-3588

46. Dahdal, R. Y., and Colley, K. J. (1993) J. Biol. Chem. 268, 26310-26319

## CLAIMS

1. A nucleic acid encoding a chimeric enzyme,  
wherein said chimeric enzyme comprises a catalytic domain  
5 of a first glycosyltransferase and a localisation signal of  
a second glycosyltransferase, whereby when said nucleic  
acid is expressed in a cell said chimeric enzyme is located  
in an area of the cell where it is able to compete for  
substrate with a second glycosyltransferase, resulting in  
10 reduced levels of a product from said second  
glycosyltransferase.
2. A nucleic acid according to claim 1, wherein said  
localisation signal localises said catalytic domain thereby  
to enable the catalytic domain to compete with said second  
15 glycosyltransferase for a substrate.
3. A nucleic acid according to claim 1 or claim 2,  
wherein the localisation signal is derived from a  
glycosyltransferase which produces glycosylation patterns  
which are recognised as foreign by a transplant recipient.
- 20 4. A nucleic acid according to any one of claims 1  
to 3, wherein the localisation signal comprises the amino  
terminus of the second glycosyltransferase.
5. A nucleic acid according to any one of claims 1  
to 4, wherein the localisation signal is derived from  
25  $\alpha(1,3)$ -galactosyltransferase.
6. A nucleic acid according to any one of claims 1  
to 5, wherein the first glycosyltransferase is selected  
from the group consisting of H-transferase, secretor  
sialyltransferase, a galactosyl sulphating enzyme or a  
30 phosphorylating enzyme.
7. A nucleic acid according to any one of claims 1  
to 6, wherein the catalytic domain and the localisation  
signal each originates from a mammal selected from the  
group consisting of human, primates, ungulates, dogs, mice,  
35 rats and rabbits.
8. A nucleic acid according to any one of claims 1  
to 7, wherein the localisation signal is derived from the



- 30 -

same species as the cell which the nucleic acid is intended to transform.

9. A nucleic acid according to any one of claims 1 to 8, comprising a sequence encoding the catalytic domain of H transferase and a nucleic acid sequence encoding a localisation signal from Gal transferase.

10. A nucleic acid according to claim 9, wherein the catalytic domain and the localisation signal are derived from pigs.

11. A nucleic acid according to any one of claims 1 to 10, which encodes gtHT as defined herein.

12. A vehicle comprising a nucleic acid according to any one of claims 1 to 11.

13. vehicle according to claim 12, selected from the group consisting of an expression vector, plasmid and phage.

14. A vehicle according to claim 12 or claim 13, which enables said nucleic acid to be expressed in prokaryotes or in eukaryotes.

15. An isolated nucleic acid molecule encoding a localisation signal of a glycosyltransferase.

16. An isolated nucleic acid molecule according to claim 15, wherein the signal encoded comprises an amino terminus of gal-transferase.

17. A method of producing a nucleic acid according to any one of claims 1 to 11, comprising the step of operably linking a nucleic acid sequence encoding a catalytic domain from a first glycosyltransferase to a nucleic acid sequence encoding a localisation signal of a second glycosyltransferase.

18. A method of reducing the level of a carbohydrate exhibited on the surface of a cell, said method comprising causing a nucleic acid to be expressed in said cell wherein said nucleic acid encodes a chimeric enzyme which comprises a catalytic domain of a first glycosyltransferase and a localisation signal of a second glycosyltransferase, whereby said chimeric enzyme is located in an area of the

cell where it is able to compete for substrate with said second glycosyltransferase, and wherein said second glycosyltransferase is capable of producing said carbohydrate.

5 19. A method of producing a cell from a donor species which is immunologically acceptable to a recipient species by reducing levels of carbohydrate on said cell which cause it to be recognised as non-self by the recipient, said method comprising causing a nucleic acid to be expressed in  
10 said cell wherein said nucleic acid encodes a chimeric enzyme which comprises a catalytic domain of a first glycosyltransferase and a localisation signal of a second glycosyltransferase, whereby said chimeric enzyme is located in an area of the cell where it is able to compete  
15 for substrate with said second glycosyltransferase, and wherein said second glycosyltransferase is capable of producing said carbohydrate.

20. A cell produced by a method according to claim 19.

20 21. An organ comprising a cell according to claim 20.

22. A non-human transgenic animal, organ or cell comprising the nucleic acid according to any one of claims 1 to 11.

23. An expression unit which expresses a nucleic acid  
25 according to any one of claims 1 to 11, resulting in a cell which is immunologically acceptable to an animal having reduced levels of a carbohydrate on its surface, which carbohydrate is recognised as non-self by said species.

24. An expression unit according to claim 23,  
30 selected from the group consisting of a retroviral-packaging cassette, retroviral construct or retroviral producer cell.

25. A method of producing an expression unit according to claim 23 or claim 24, said unit having reduced  
35 levels of a carbohydrate on its surface wherein the carbohydrate is recognised as non-self by a species, comprising transforming/transfecting a retroviral packaging

- 32 -

cell or a retroviral producer cell with the nucleic acid of the invention under conditions such that the chimeric enzyme is produced.

1/12

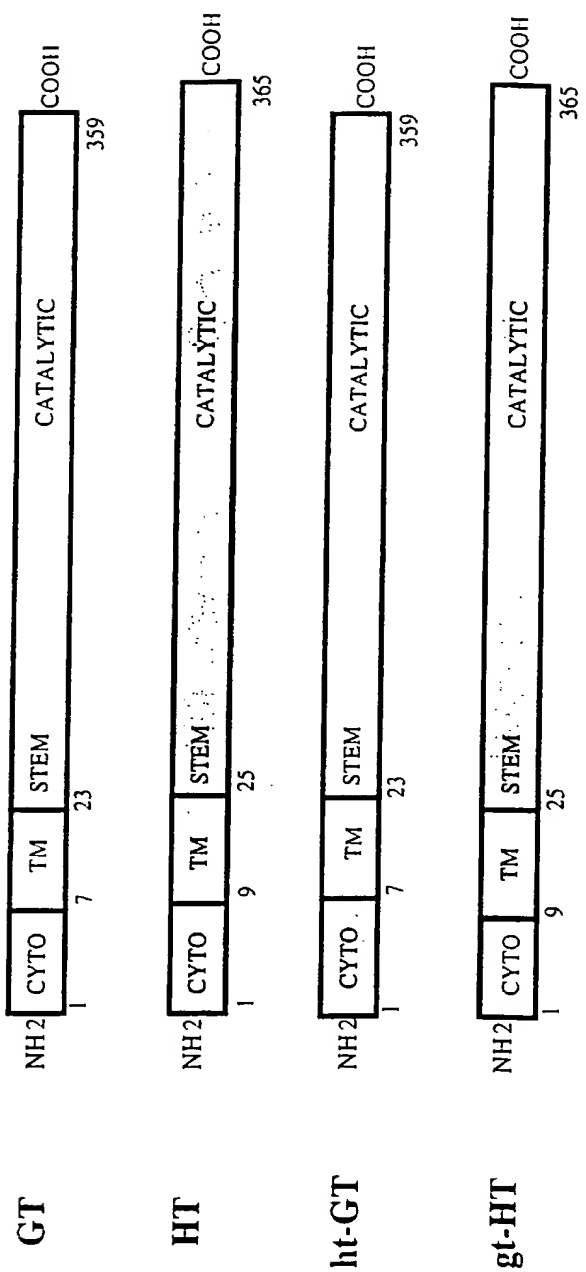


FIGURE 1

SUBSTITUTE SHEET (Rule 26)

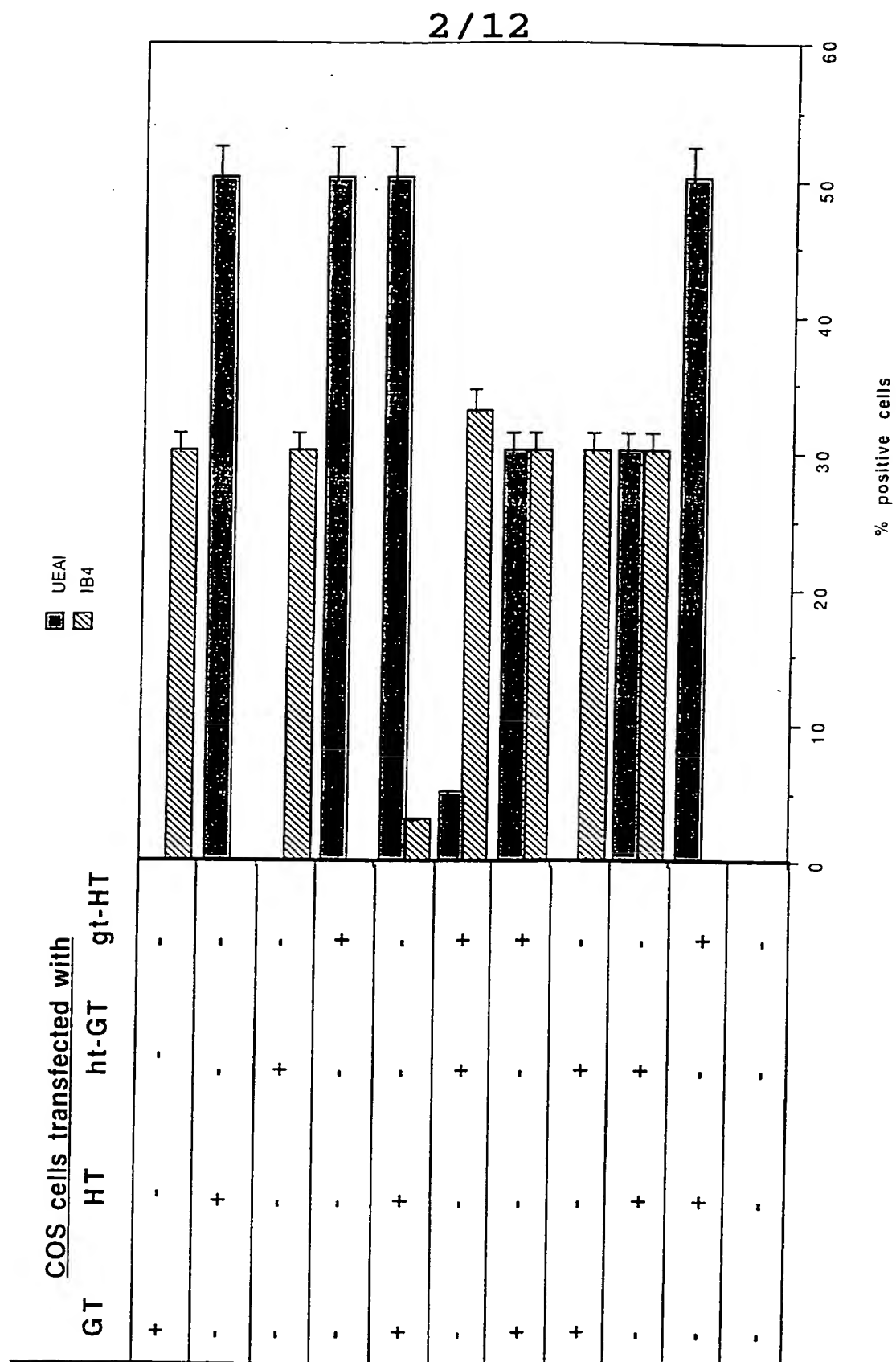


FIGURE 2

3 / 12

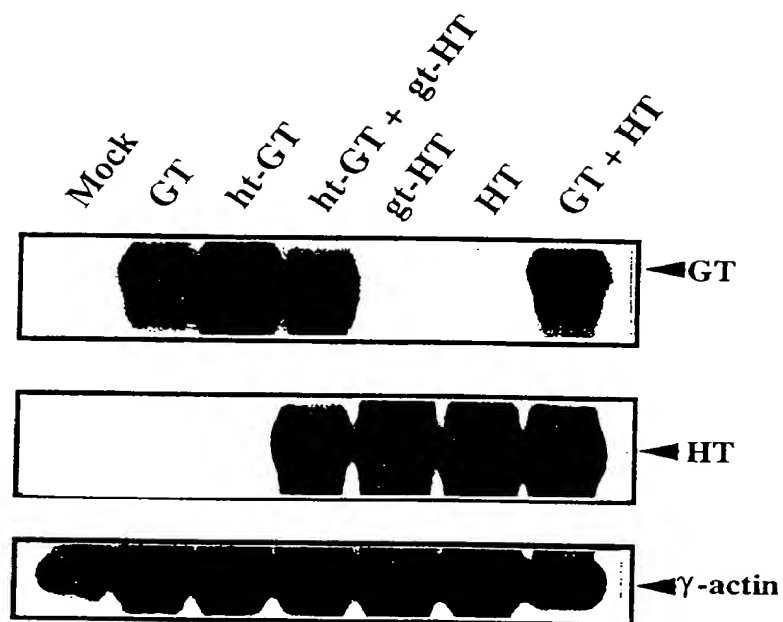


FIGURE 3

4/12

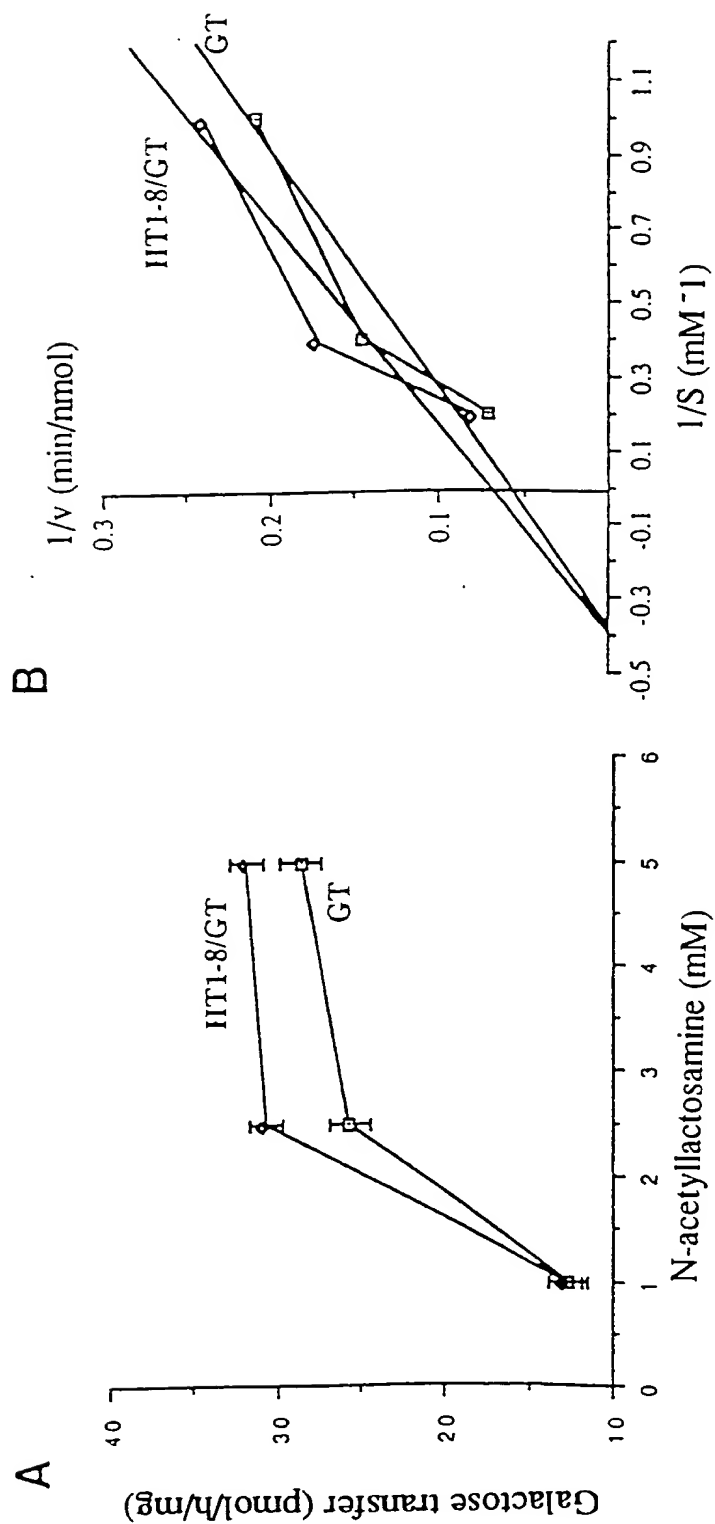


FIGURE 4

5/12

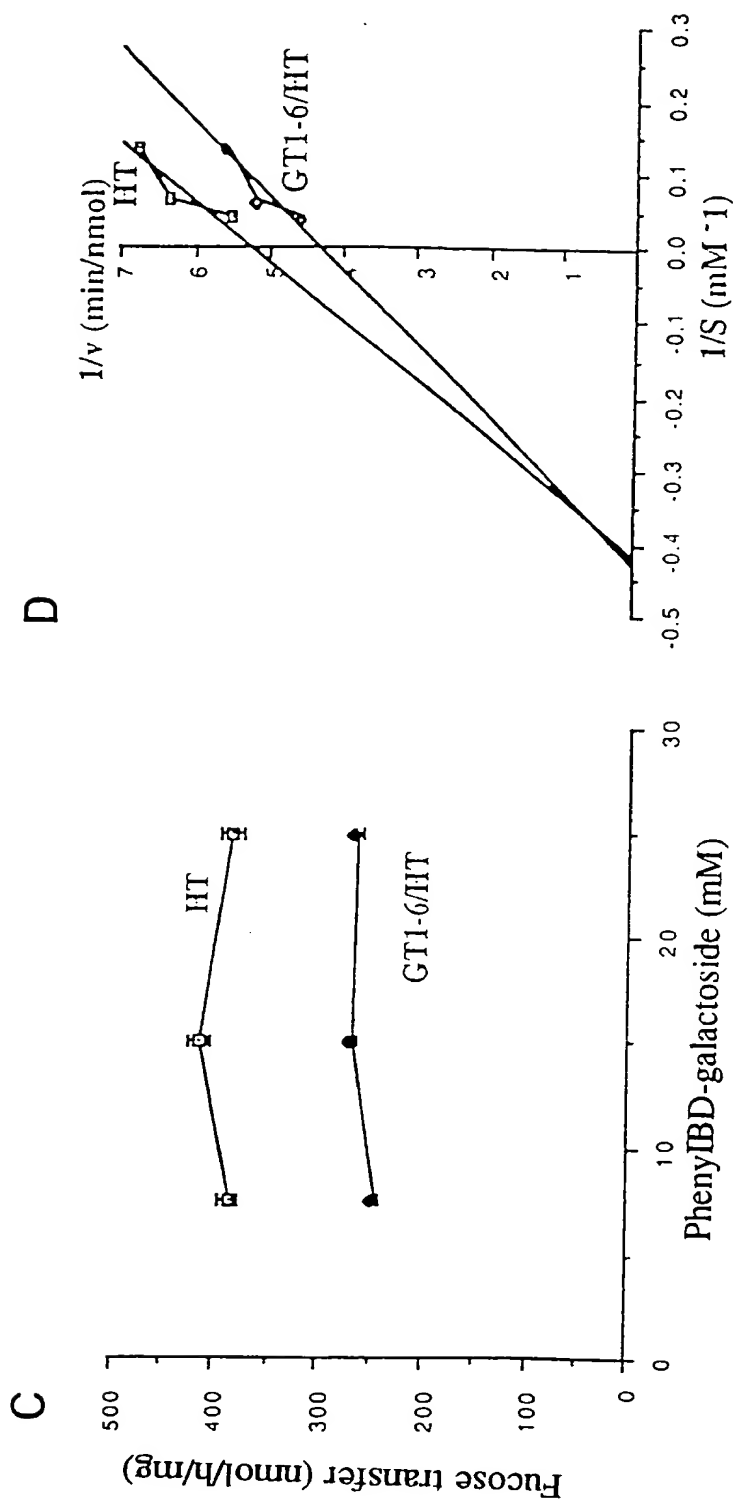


FIGURE 4 Continued

SUBSTITUTE SHEET (RULE 26)



6/12

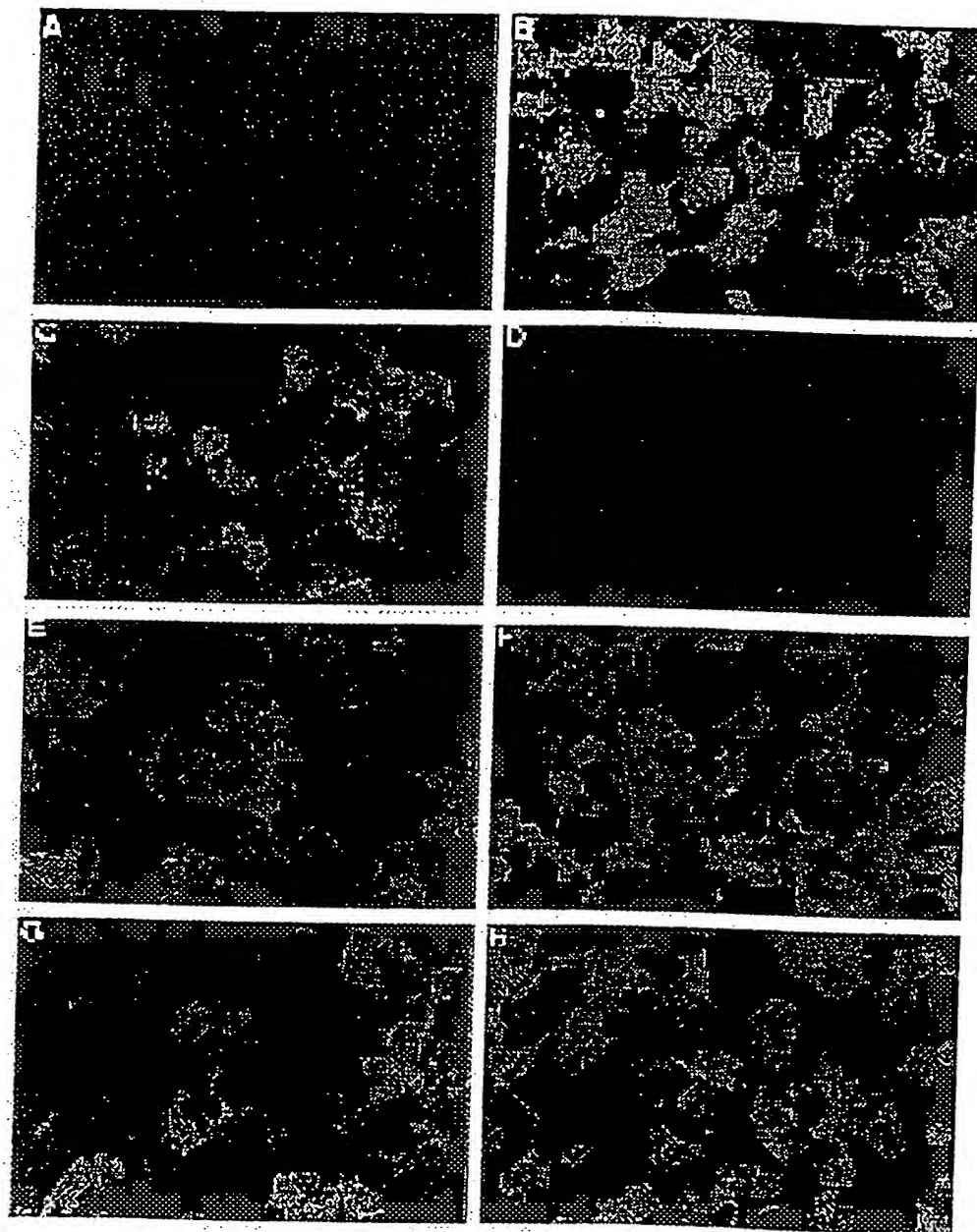


FIGURE 5

SUBSTITUTE SHEET (RULE 26)

7/12

## PORCINE SECRETOR SEQUENCE

M L S M Q A S F F F P T G P F I L 17  
 CT ACA GCC ATG CTC AGC ATG CAG GCA TCC TTC TTC CCC ACG GGT CCC TTC ATC CTC 59  
 F V F T A S T I F H L Q Q R M V K I Q P 37  
 TTT GTC TTC ACG GCT TCC ACC ATA TTT CAC CTT CAG CAG AGG ATG GTG AAG ATT CAA CCC 119  
 T W E L Q M V T Q V T T E S P S P Q L 57  
 ACG TGG GAG TTA CAG ATG GTG ACG CAG GTG ACC ACA GAG AGC CCC TCG AGC CCC CAG CTG 179

## PORCINE SECRETOR SEQUENCE

K G M W T I N A I G R L G N Q M G E Y A 77  
 AAG GGC ATG TGG ACG ATC AAT GCC ATC GGC CGC CTG GGC AAC CAG ATG GGG GAG TAC GCC 239  
 T L Y A L A R M N G R P A F I P P E M H 97  
 ACC CTG TAC GCG CTG GCC AGG ATG AAC GGG CGG CGG GCC TTC ATC CCG CCC GAG ATG CAC 299  
 S T L A P I F R I T L P V L H A S T A R 117  
 AGC ACG CTG GCC CCC ATC TTC AGG ATC ACC CTC CCG GTC CTG CAC GCC AGC ACG GCC CGC 359  
 R I P W Q N Y H L N D W M E E R Y R H I 137  
 AGG ATC CCC TGG CAG AAC TAC CAC CTG AAC GAC TGG ATG GAG GAG CGG TAC CGC CAC ATC 419  
 P G E Y V R L T G Y P C S W T F Y H L 157

FIGURE 6

SUBSTITUTE SHEET (RULE 26)

8/12

CCG GGG GAG TAC GTG CGC CTC ACG GGC TAC CCC TGC TCC TGG ACC TTC TAC CAC CAC CTG 479  
 R T E I L R E F T L H N H V R E E A Q D 177  
 CGC ACC GAG ATC CTC CGG GAG TTC ACC CTG CAT AAC CAC GTG CGC GAG GAG GCC CAG GAT 539  
 F L R G L R V N G S R P S T Y V G V H V 197  
 TTC CTG CGG GGT CTG CGG GTG AAC GGG AGC CGA CCG AGT ACC TAC GTG GGG GTG CAC GTG 599  
 R R G D Y V H V M P N V W K G V A D R 217  
 CGC CGG GGG GAC TAC GTG CAC GTG ATG CCC AAC GTG TGG AAG GGC GTG GCC GAC CGG 659  
 R Y L E Q A L D W F R A R Y R S P V F V 237  
 CGG TAC CTG GAG CAG GCC CTG GAC TGG TTC CGG GCT CGC TAC CGC TCC CCC GTC TTT GTG 719  
 V S S N G M A W C R E N I N A S R G D V 257  
 GTC TCC AGC AAC GGC ATG GCC TGG TGT CGG GAA AAC ATC AAT GCC TCG CGC GGC GAT GTG 779  
 V F A G N G I E G S P A K D F A L L T Q 277  
 GTG TTT GCC GGC AAT GGC ATC GAG GGC TCC CCC GCC AAA GAC TTC GCG CTG CTC ACG CAG 839  
 C N H T V M T I G T F G I W A A Y L A G 297  
 TGT AAC CAC ACT GTC ATG ACC ATT GGC ACG TTC GGG ATC TGG GCC GCC TAC CTT GCT GGT 899  
 G E T I Y L A N Y T L P D S P F L K L F 317  
 GGA GAG ACC ATC TAC CTG GCC AAT TAC ACG CTC CCG GAC TCT CCC TTC CTC AAA CTC TTT 959

FIGURE 6 (cont.)

SUBSTITUTE SHEET (RULE 26)

9/12

K P E A A F L P E W I G I E A D L S P L 337  
AAG CCC GAG GCA GCC TTC CTG CCC GAG TGG ATT GGG ATC GAG GCA GAC CTG TCC CCA CTC 1019

L K H \* 340  
CTT AAG CAC TGA TGT CGG CTG TCC 1043

FIGURE 6 (cont.)

SUBSTITUTE SHEET (RULE 26)

## 10/12

## PIG H TRANSFERASE

M W V P S R R H L C L T F L - L V C V L A	20
ATGTGGGTCCCCAGCCGCCGCCACCTCTGTCTGACCTTCCTGCTAGTCTGTGTTTTAGCA	60
A I F F L N V Y Q D L F Y S G L D L L A	40
GCAATTTTCTTCCTGAACGTCTATCAAGACCTCTTTTACAGTGGCTTAGACCTGCTGGCC	120
L C P D H N V V S S P V A I F C L A G T	60
CTGTGTCCAGACCATAACCTGGTATCATCTCCCGTGGCCATATTCTGCCTGGCGGGCAGC	180
P V H P N A S D S C P K H P A S F S G T	80
CCGGTACACCCCAACGCCTCCGATTCTGTCTCCAAGCATCTGCCTCCTTTTCCGGGACC	240
W T I Y P D G R F G N Q M G Q Y A T L L	100
TGGACTATTTACCCGGATGGCCGGTTTGGGAACCAGATGGGACAGTATGCCACGCTGCTG	300
A L A Q L N G R Q A F I Q P A M H A V L	120
GCCCTGGCGCAGCTCAACGGCCGCCAGGCCTTCATCCAGCCTGCCATGCACGCCGTCCTG	360
A P V F R I T L P V L A P E V D R H A P	140
GCCCCCGTGTTCGGCATCAGCGTGCCTGTCTGGCGCCCGAGGTAGACAGGCACGCTCCT	420
W R E L E L E D W M S E D Y A H L K E P	160
TGGCGGGAGCTGGAGCTTCACGACTGGATGTCCGAGGATTATGCCCACTTAAAGGAGCCC	480
W L K L T G F P C S W T F F H H L R E Q	180
TGGCTGAAGCTCACCGGCTTCCCCGTGCTCCTGGACCTTCTTCCACCACCTCCGGGAGCAG	540
I R S E F T L H D H L R Q E A Q G V L S	200
ATCCGCAGCGAGTTACCCCTGCACGACCACCTTCGGCAAGAGGCCACAGGGGGTACTGAGT	600
Q F R L P R T G D R P S T F V G V H V R	220
CAGTTCCGTCTACCCCGCACAGGGGACCGCCCCAGCACCTTCGTGGGGGTCCACGTGCGC	660
R G D Y L R V M P K R W K G V V G D G A	240
CGCGGGGACTATCTGCGTGTGATGCCCAAGCGCTGGAAGGGGGTGGTGGGTGACGGCCCT	720
Y L Q Q A M D W F R A R V E A P V F V V	260
TACCTCCAGCAGGCTATGGACTGGTTCCGGGGCCCGATACGAAGCCCCGTCTTTGTGGTC	780
T S N G M E W C R K N I D T S R G D V I	280
ACCAGCAACGGCATGGAGTGGTGCCGGAAGAACATCGACACCTCCCGGGGGGACGTGATC	840
F A G D G R E A A P A R D F A L L V Q C	300
TTTGCTGGCGATGGGCGGGAGGCCCGCCCGCCAGGGACTTTGCGCTGCTGGTGCAGTGC	900
N H T I M T I G T F G F W A A Y L A G G	320
AACCACACCATCATGACCATTTGGCACCTTCGGCTTCTGGGGCCGCTACCTGGCTGGTGGA	960
D T I Y L A N F T L P T S S F L K I F K	340
GATACCATCTACTTGGCTAACTTCACCTGCCCACTTCCAGCTTCCTGAAGATCTTTAA	1020
P E A A F L P E W V G I N A D L S P L Q	360
CCCGAGGCTGCCTTCCTCCCGAGTGGGTGGGCATTAATGCAGACTTGTCTCCACTCCAG	1080
M L A G P *	365
ATGTTGGCTGGGCCTTGA	1098

## FIGURE 7

SUBSTITUTE SHEET (RULE 26)

11/12

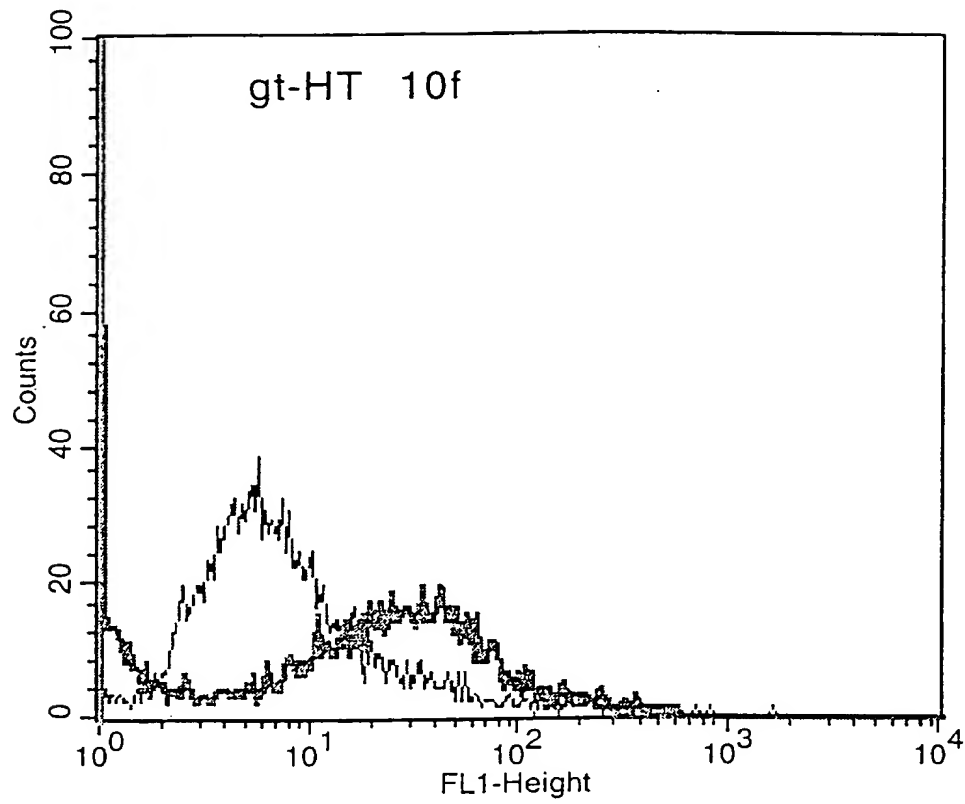


FIGURE 8

SUBSTITUTE SHEET (RULE 26)

12/12



FIGURE 9

SUBSTITUTE SHEET (RULE 26)

**INTERNATIONAL SEARCH REPORT**

International Application No.

**PCT/AU 97/00492****A. CLASSIFICATION OF SUBJECT MATTER**Int Cl<sup>B</sup>: C12N 15/11, 15/62, 5/16, 15/54

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

WPAT C12N 15/62 AND 15/54 and Chemical Abstracts see below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
STN:Medline see below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPAT C12N 15/62 AND (ENZYME# OR TRANSFERASE#) C12N 15/54, AND (CHIMER:OR CHIMAER:OR FUSION# OR MUTANT# OR HYBRID#) for STN and Chem Abs see attached

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nature Medicine 1 (12) pages 1261-67 (1995) Sandrin, M. S. et al "Enzymatic remodelling of the carbohydrate surface of a xenogenic cell substantially reduces human antibody binding and complement-mediated cytotoxicity"	

Further documents are listed in the  
continuation of Box C

See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

Date of mailing of the international search report

**22 OCT 1997**Name and mailing address of the ISA/AU  
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION  
PO BOX 200  
WODEN ACT 2606  
AUSTRALIA Facsimile No.: (02) 6285 3929

Authorized officer

**JIM CHAN**

Telephone No.: (02) 6283 2340



# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 97/00492

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A, 94 12646 (CIBA GEIGY AC) 9 June 1994	
A	J. Biol. Chem. 269 (16) pages 12049-59 (1994) Burke, J. et al "Medial-Golgi Retention of N-Acetylglucosaminyl transferase I".	
PX	J. Biol. Chem. (1996) 271 (51) pages 33105-33109. Osman, N. et al "Switching Amino-terminal cytoplasmic Domains of $\alpha$ (1, 2) Fucosyltransferase and $\alpha$ (1, 3) Galactosyltransferase Alters the Expression of H Substance and Gal $\alpha$ (1, 3) Gal.	

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 97/00492

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 97/00492

## Box

Continuation of **FIELDS SEARCHED BOX B** Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN MEDLINE (glycosyltransferase/CT OR galactosyltransferase/CT OR sialyltransferase/CT OR fucosyltransferase/CT OR glucosyltransferase/CT) AND (chimera or chimeric or fusion or hybrid)

CHEM ABS (glycosyltransferase/IT OR galactosyltransferase/IT OR sialyltransferase/IT OR fucosyltransferase/IT OR glucosyltransferase/IT) AND (chimera or chimeric or fusion or hybrid) AND 7-5/ccError! Bookmark not defined.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International Application No.  
**PCT/AU 97/00492**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report			Patent Family Member
WO	9412646	AU	940622
		CA	940609
		EP	970813
		FI	950526
		MX	940831
		NO	950523
		US	970624
		WO	940609
END OF ANNEX			